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(54) Title: **DIAGNOSIS OF DISEASES ASSOCIATED WITH SIGNAL TRANSDUCTION**

CG TG



I

CG TG



II

(57) Abstract: The present invention relates to the chemically modified genomic sequences of genes associated with signal transduction, to oligonucleotides and/or PNA-oligomers for detecting the cytosine methylation state of genes associated with signal transduction which are directed against the sequence, as well as to a method for ascertaining genetic and/or epigenetic parameters of genes associated with signal transduction.

WO 02/00926 A2

Diagnosis of Diseases Associated with signal transduction

Field of the Invention

The levels of observation that have been well studied by the methodological developments of recent years in molecular biology, are the genes themselves, the translation of these genes into RNA, and the resulting proteins. The question of which gene is switched on at which point in the course of the development of an individual, and how the activation and inhibition of specific genes in specific cells and tissues are controlled is correlatable to the degree and character of the methylation of the genes or of the genome. In this respect, pathogenic conditions may manifest themselves in a changed methylation pattern of individual genes or of the genome.

The present invention relates to nucleic acids, oligonucleotides, PNA-oligomers and to a method for the diagnosis and/or therapy of diseases which have a connection with the genetic and/or epigenetic parameters of genes associated with signal transduction and, in particular, with the methylation status thereof.

Prior Art

Eukaryotic cells exist in a social context, as such, they require a system that allows inter cellular communication. Molecules such as hormones, growth factors and neurotransmitters are utilised as transmitters in cell signalling. These signals allow for the adjustment of factors such as metabolism, growth, proliferation and apoptosis that are essential to the participation of the cell in a social environment. Signal transduction can be defined as the movement of such signals from outside the cell to inside the cell. Signal transmission may be simple, as in the case of acetylcholine receptors which allow the movement of signals through ion channels on the plasma membrane surface. Alternatively, the signals may be transmitted in a more complicated manner through an intracellular signalling cascade by means of protein phosphorylation, (the addition and removal of phosphate groups by protein kinases and protein phosphatases). The system has evolved a high degree of specificity whereby the kinases and phosphatases of the system are extraordinarily precise with respect to their substrates. Signals received at the cell membrane stimulate the activity of a signal integrating complex, which then interacts with substrates, finally

leading to a phenotypic response. Furthermore, evidence is emerging that protein kinases and protein phosphatases work in concert to moderate precise, fast signal transduction that is regulated by feedback.

Signal receptors can be classified into three classes. The first class consists of receptors that span the plasma membrane and which have intrinsic enzymatic activity, such as, tyrosine kinases and guanylate cyclases. The second group consists of, receptors that are coupled to GTP-binding and hydrolyzing proteins, such as odorant receptors and some hormone receptors. The third group comprises intracellular receptors that directly affect gene transcription upon ligand binding.

Disruptions to cell signalling pathways are implicated in many diseases, including cancer, immune disease and inflammatory disorders. Therefore, the elucidation of cell signalling pathways is of considerable importance. The signalling pathways are currently far from completely understood. However, this has not deterred the use of signal transduction as a drug discovery platform, all major pharmaceutical companies currently pursue active drug discovery programs based on signal transduction.

5-methylcytosine is the most frequent covalent base modification in the DNA of eukaryotic cells. It plays a role, for example, in the regulation of the transcription, in genetic imprinting, and in tumorigenesis.

Aberrant DNA methylation within CpG islands is common in human malignancies leading to abrogation or overexpression of a broad spectrum of genes (Jones, P.A. *Cancer Res* 65:2463-2467, 1996). Abnormal methylation has also been shown to occur in CpG rich regulatory elements in intronic and coding parts of genes for certain tumours (Chan, M.F., et al., *Curr Top Microbiol Immunol* 249:75-86, 2000). Using restriction landmark genomic scanning, Costello and coworkers were able to show that methylation patterns are tumour-type specific (Costello, J. F., et al., *Nat Genet* 24:132-138, 2000). Highly characteristic DNA methylation patterns could also be shown for breast cancer cell lines (Huang, T. H.-M., et al., *Hum Mol Genet* 8:459-470, 1999). Genome wide assessment of methylation status represents a molecular fingerprint of cancer tissues.

Therefore, the identification of 5-methylcytosine as a component of genetic information is of considerable interest. However, 5-methylcytosine positions cannot be identified by sequencing since 5-methylcytosine has the same base pairing behavior as cytosine. Moreover, the epigenetic information carried by 5-methylcytosine is completely lost during PCR amplification.

A relatively new and currently the most frequently used method for analyzing DNA for 5-methylcytosine is based upon the specific reaction of bisulfite with cytosine which, upon subsequent alkaline hydrolysis, is converted to uracil which corresponds to thymidine in its base pairing behavior. However, 5-methylcytosine remains unmodified under these conditions. Consequently, the original DNA is converted in such a manner that methylcytosine, which originally could not be distinguished from cytosine by its hybridization behavior, can now be detected as the only remaining cytosine using "normal" molecular biological techniques, for example, by amplification and hybridization or sequencing. All of these techniques are based on base pairing which can now be fully exploited. In terms of sensitivity, the prior art is defined by a method which encloses the DNA to be analyzed in an agarose matrix, thus preventing the diffusion and renaturation of the DNA (bisulfite only reacts with single-stranded DNA), and which replaces all precipitation and purification steps with fast dialysis (Olek A, Oswald J, Walter J. A modified and improved method for bisulphite based cytosine methylation analysis. *Nucleic Acids Res.* 1996 Dec 15;24(24):5064-6). Using this method, it is possible to analyze individual cells, which illustrates the potential of the method. However, currently only individual regions of a length of up to approximately 3000 base pairs are analyzed, a global analysis of cells for thousands of possible methylation events is not possible. However, this method cannot reliably analyze very small fragments from small sample quantities either. These are lost through the matrix in spite of the diffusion protection.

An overview of the further known methods of detecting 5-methylcytosine may be gathered from the following review article: Rein, T., DePamphilis, M. L., Zorbas, H., *Nucleic Acids Res.* 1998, 26, 2255.

To date, barring few exceptions (e.g., Zeschnick M, Lich C, Buiting K, Doerfler W, Horsthemke B. A single-tube PCR test for the diagnosis of Angelman and Prader-Willi syndrome based on allelic methylation differences at the SNRPN locus. *Eur J Hum Genet.* 1997 Mar-Apr;5(2):94-8)

the bisulfite technique is only used in research. Always, however, short, specific fragments of a known gene are amplified subsequent to a bisulfite treatment and either completely sequenced (Olek A, Walter J. The pre-implantation ontogeny of the H19 methylation imprint. *Nat Genet.* 1997 Nov;17(3):275-6) or individual cytosine positions are detected by a primer extension reaction (Gonzalzo ML, Jones PA. Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). *Nucleic Acids Res.* 1997 Jun 15;25(12):2529-31, WO 95/0669) or by enzymatic digestion (Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res.* 1997 Jun 15;25(12):2532-4). In addition, detection by hybridization has also been described (Olek et al., WO 99/8498).

Further publications dealing with the use of the bisulfite technique for methylation detection in individual genes are: Grigg G, Clark S. Sequencing 5-methylcytosine residues in genomic DNA. *Bioessays.* 1994 Jun;16(6):431-6, 431; Zeschnigk M, Schmitz B, Dittrich B, Buiting K, Horsthemke B, Doerfler W. Imprinted segments in the human genome: different DNA methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic sequencing method. *Hum Mol Genet.* 1997 Mar;6(3):387-95; Feil R, Charlton J, Bird AP, Walter J, Reik W. Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing. *Nucleic Acids Res.* 1994 Feb 25;22(4):695-6; Martin V, Ribieras S, Song-Wang X, Rio MC, Dante R. Genomic sequencing indicates a correlation between DNA hypomethylation in the 5' region of the pS2 gene and its expression in human breast cancer cell lines. *Gene.* 1995 May 19;157(1-2):261-4; WO 97/6705, WO 95/5373 and WO 97/45560.

An overview of the Prior Art in oligomer array manufacturing can be gathered from a special edition of *Nature Genetics* (*Nature Genetics Supplement*, Volume 21, January 1999), published in January 1999, and from the literature cited therein.

Fluorescently labeled probes are often used for the scanning of immobilized DNA arrays. The simple attachment of Cy3 and Cy5 dyes to the 5'-OH of the specific probe are particularly suitable for fluorescence labels. The detection of the fluorescence of the hybridized probes may be carried out, for example via a confocal microscope. Cy3 and Cy5 dyes, besides many others, are commercially available.

Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-TOF) is a very efficient development for the analysis of biomolecules (Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal Chem.* 1988 Oct 15;60(20):2299-301). An analyte is embedded in a light-absorbing matrix. The matrix is evaporated by a short laser pulse thus transporting the analyte molecule into the vapor phase in an unfragmented manner. The analyte is ionized by collisions with matrix molecules. An applied voltage accelerates the ions into a field-free flight tube. Due to their different masses, the ions are accelerated at different rates. Smaller ions reach the detector sooner than bigger ones.

MALDI-TOF spectrometry is excellently suited to the analysis of peptides and proteins. The analysis of nucleic acids is somewhat more difficult (Gut I G, Beck S. DNA and Matrix Assisted Laser Desorption Ionization Mass Spectrometry. *Current Innovations and Future Trends.* 1995, 1; 147-57). The sensitivity to nucleic acids is approximately 100 times worse than to peptides and decreases disproportionally with increasing fragment size. For nucleic acids having a multiply negatively charged backbone, the ionization process via the matrix is considerably less efficient. In MALDI-TOF spectrometry, the selection of the matrix plays an eminently important role. For the desorption of peptides, several very efficient matrixes have been found which produce a very fine crystallization. There are now several responsive matrixes for DNA, however, the difference in sensitivity has not been reduced. The difference in sensitivity can be reduced by chemically modifying the DNA in such a manner that it becomes more similar to a peptide. Phosphorothioate nucleic acids in which the usual phosphates of the backbone are substituted with thiophosphates can be converted into a charge-neutral DNA using simple alkylation chemistry (Gut IG, Beck S. A procedure for selective DNA alkylation and detection by mass spectrometry. *Nucleic Acids Res.* 1995 Apr 25;23(8):1367-73). The coupling of a charge tag to this modified DNA results in an increase in sensitivity to the same level as that found for peptides. A further advantage of charge tagging is the increased stability of the analysis against impurities which make the detection of unmodified substrates considerably more difficult.

Genomic DNA is obtained from DNA of cell, tissue or other test samples using standard methods. This standard methodology is found in references such as Fritsch and Maniatis eds., *Molecular Cloning: A Laboratory Manual*, 1989.

Description

The object of the present invention is to provide the chemically modified DNA of genes associated with signal transduction, as well as oligonucleotides and/or PNA-oligomers for detecting cytosine methylations, as well as a method which is particularly suitable for the diagnosis and/or therapy of genetic and epigenetic parameters of genes associated with signal transduction. The present invention is based on the discovery that genetic and epigenetic parameters and, in particular, the cytosine methylation pattern of genes associated with signal transduction are particularly suitable for the diagnosis and/or therapy of diseases associated with signal transduction.

This objective is achieved according to the present invention using a nucleic acid containing a sequence of at least 18 bases in length of the chemically pretreated DNA of genes associated with signal transduction according to one of Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto. In the table, after the listed gene designations, the respective data bank numbers (accession numbers) are specified which define the appertaining gene sequences as unique. GenBank was used as the underlying data bank which is located at internet address <http://www.ncbi.nlm.nih.gov>.

The chemically modified nucleic acid could heretofore not be connected with the ascertainment of genetic and epigenetic parameters.

The object of the present invention is further achieved by an oligonucleotide or oligomer for detecting the cytosine methylation state in chemically pretreated DNA, containing at least one base sequence having a length of at least 13 nucleotides which hybridizes to a chemically pretreated DNA of genes associated with signal transduction according to Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto. The oligomer probes according to the present invention constitute important and effective tools which, for the first time, make it possible to ascertain the genetic and epigenetic parameters of genes associated with signal transduction. The base sequence of the oligomers preferably contains at least one CpG dinucleotide. The probes may also exist in the form of a PNA (peptide nucleic acid) which has particularly preferred pairing properties. Particularly preferred are oligonucleotides according to the present invention in which the cytosine of the CpG dinucleotide is the 5th - 9th nucleotide from

the 5'-end of the 13-mer; in the case of PNA-oligomers, it is preferred for the cytosine of the CpG dinucleotide to be the 4th - 6th nucleotide from the 5'-end of the 9-mer.

The oligomers according to the present invention are normally used in so called "sets" which contain at least one oligomer for each of the CpG dinucleotides of the sequences of Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto. Preferred is a set which contains at least one oligomer for each of the CpG dinucleotides from one of Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto.

Moreover, the present invention makes available a set of at least two oligonucleotides which can be used as so-called "primer oligonucleotides" for amplifying DNA sequences of one of Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto, or segments thereof.

In the case of the sets of oligonucleotides according to the present invention, it is preferred that at least one oligonucleotide is bound to a solid phase.

The present invention moreover relates to a set of at least 10 n (oligonucleotides and/or PNA-oligomers) used for detecting the cytosine methylation state in chemically pretreated genomic DNA (Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto). These probes enable diagnosis and/or therapy of genetic and epigenetic parameters of genes associated with signal transduction. The set of oligomers may also be used for detecting single nucleotide polymorphisms (SNPs) in the chemically pretreated DNA of genes associated with signal transduction according to one of Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto.

According to the present invention, it is preferred that an arrangement of different oligonucleotides and/or PNA-oligomers (a so-called "array") made available by the present invention is pre-

sent in a manner that it is likewise bound to a solid phase. This array of different oligonucleotide- and/or PNA-oligomer sequences can be characterized in that it is arranged on the solid phase in the form of a rectangular or hexagonal lattice. The solid phase surface is preferably composed of silicon, glass, polystyrene, aluminum, steel, iron, copper, nickel, silver, or gold. However, nitrocellulose as well as plastics such as nylon which can exist in the form of pellets or also as resin matrices are possible as well.

Therefore, a further subject matter of the present invention is a method for manufacturing an array fixed to a carrier material for analysis in connection with diseases associated with signal transduction in which method at least one oligomer according to the present invention is coupled to a solid phase. Methods for manufacturing such arrays are known, for example, from US Patent 5,744,305 by means of solid-phase chemistry and photolabile protecting groups.

A further subject matter of the present invention relates to a DNA chip for the analysis of diseases associated with signal transduction which contains at least one nucleic acid according to the present invention. DNA chips are known, for example, for US Patent 5,837,832.

Moreover, a subject matter of the present invention is a kit which may be composed, for example, of a bisulfite-containing reagent, a set of primer oligonucleotides containing at least two oligonucleotides whose sequences in each case correspond or are complementary to an 18 base long segment of the base sequences specified in the appendix (Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto), oligonucleotides and/or PNA-oligomers as well as instructions for carrying out and evaluating the described method. However, a kit along the lines of the present invention can also contain only part of the aforementioned components.

The present invention also makes available a method for ascertaining genetic and/or epigenetic parameters of genes associated with the cycle cell by analyzing cytosine methylations and single nucleotide polymorphisms, including the following steps:

In the first step of the method, a genomic DNA sample is chemically treated in such a manner that cytosine bases which are unmethylated at the 5'-position are converted to uracil, thymine, or

another base which is dissimilar to cytosine in terms of hybridization behavior. This will be understood as 'chemical pretreatment' hereinafter.

The genomic DNA to be analyzed is preferably obtained from usual sources of DNA such as cells or cell components, for example, cell lines, biopsies, blood, sputum, stool, urine, cerebrospinal fluid, tissue embedded in paraffin such as tissue from eyes, intestine, kidney, brain, heart, prostate, lung, breast or liver, histologic object slides, or combinations thereof.

The above described treatment of genomic DNA is preferably carried out with bisulfite (hydrogen sulfite, disulfite) and subsequent alkaline hydrolysis which results in a conversion of non-methylated cytosine nucleobases to uracil or to another base which is dissimilar to cytosine in terms of base pairing behavior.

Fragments of the chemically pretreated DNA are amplified, using sets of primer oligonucleotides according to the present invention, and a, preferably heat-stable polymerase. Because of statistical and practical considerations, preferably more than ten different fragments having a length of 100 - 2000 base pairs are amplified. The amplification of several DNA segments can be carried out simultaneously in one and the same reaction vessel. Usually, the amplification is carried out by means of a polymerase chain reaction (PCR).

In a preferred embodiment of the method, the set of primer oligonucleotides includes at least two oligonucleotides whose sequences are each reverse complementary or identical to an at least 18 base-pair long segment of the base sequences specified in the appendix (Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto). The primer oligonucleotides are preferably characterized in that they do not contain any CpG dinucleotides.

According to the present invention, it is preferred that at least one primer oligonucleotide is bonded to a solid phase during amplification. The different oligonucleotide and/or PNA-oligomer sequences can be arranged on a plane solid phase in the form of a rectangular or hexagonal lattice, the solid phase surface preferably being composed of silicon, glass, polystyrene, aluminum, steel, iron, copper, nickel, silver, or gold, it being possible for other materials such as nitrocellulose or plastics to be used as well.

The fragments obtained by means of the amplification can carry a directly or indirectly detectable label. Preferred are labels in the form of fluorescence labels, radionuclides, or detachable molecule fragments having a typical mass which can be detected in a mass spectrometer, it being preferred that the fragments that are produced have a single positive or negative net charge for better detectability in the mass spectrometer. The detection may be carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

The amplicates obtained in the second step of the method are subsequently hybridized to an array or a set of oligonucleotides and/or PNA probes. In this context, the hybridization takes place in the manner described in the following. The set of probes used during the hybridization is preferably composed of at least 10 oligonucleotides or PNA-oligomers. In the process, the amplicates serve as probes which hybridize to oligonucleotides previously bonded to a solid phase. The non-hybridized fragments are subsequently removed. Said oligonucleotides contain at least one base sequence having a length of 13 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the appendix, the segment containing at least one CpG dinucleotide. The cytosine of the CpG dinucleotide is the 5th to 9th nucleotide from the 5'-end of the 13-mer. One oligonucleotide exists for each CpG dinucleotide. Said PNA-oligomers contain at least one base sequence having a length of 9 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the appendix, the segment containing at least one CpG dinucleotide. The cytosine of the CpG dinucleotide is the 4th to 6th nucleotide seen from the 5'-end of the 9-mer. One oligonucleotide exists for each CpG dinucleotide.

In the fourth step of the method, the non-hybridized amplicates are removed.

In the final step of the method, the hybridized amplicates are detected. In this context, it is preferred that labels attached to the amplicates are identifiable at each position of the solid phase at which an oligonucleotide sequence is located.

According to the present invention, it is preferred that the labels of the amplicates are fluorescence labels, radionuclides, or detachable molecule fragments having a typical mass which can

be detected in a mass spectrometer. The mass spectrometer is preferred for the detection of the amplicates, fragments of the amplicates or of probes which are complementary to the amplicates, it being possible for the detection to be carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

The produced fragments may have a single positive or negative net charge for better detectability in the mass spectrometer. The aforementioned method is preferably used for ascertaining genetic and/or epigenetic parameters of genes associated with signal transduction.

The oligomers according to the present invention or arrays thereof as well as a kit according to the present invention are intended to be used for the diagnosis and/or therapy of diseases associated with signal transduction by analyzing methylation patterns of genes associated with signal transduction. According to the present invention, the method is preferably used for the diagnosis and/or therapy of important genetic and/or epigenetic parameters within genes associated with signal transduction.

The method according to the present invention is used, for example, for the diagnosis and/or therapy of solid tumours and cancer. The nucleic acids according to the present invention of Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto can be used for the diagnosis and/or therapy of genetic and/or epigenetic parameters of genes associated with signal transduction.

The present invention moreover relates to a method for manufacturing a diagnostic agent and/or therapeutic agent for the diagnosis and/or therapy of diseases associated with signal transduction by analyzing methylation patterns of genes associated with signal transduction, the diagnostic agent and/or therapeutic agent being characterized in that at least one nucleic acid according to the present invention is used for manufacturing it, possibly together with suitable additives and auxiliary agents.

A further subject matter of the present invention relates to a diagnostic agent and/or therapeutic agent for diseases associated with signal transduction by analyzing methylation patterns of genes

associated with signal transduction, the diagnostic agent and/or therapeutic agent containing at least one nucleic acid according to the present invention, possibly together with suitable additives and auxiliary agents.

The present invention moreover relates to the diagnosis and/or prognosis of events which are disadvantageous to patients or individuals in which important genetic and/or epigenetic parameters within genes associated with signal transduction said parameters obtained by means of the present invention may be compared to another set of genetic and/or epigenetic parameters, the differences serving as the basis for a diagnosis and/or prognosis of events which are disadvantageous to patients or individuals.

In the context of the present invention the term "hybridization" is to be understood as a bond of an oligonucleotide to a completely complementary sequence along the lines of the Watson-Crick base pairings in the sample DNA, forming a duplex structure. To be understood by "stringent hybridization conditions" are those conditions in which a hybridization is carried out at 60°C in 2.5 x SSC buffer, followed by several washing steps at 37°C in a low buffer concentration, and remains stable.

The term "functional variants" denotes all DNA sequences which are complementary to a DNA sequence, and which hybridize to the reference sequence under stringent conditions and have an activity similar to the corresponding polypeptide according to the present invention.

In the context of the present invention, "genetic parameters" are mutations and polymorphisms of genes associated with signal transduction and sequences further required for their regulation. To be designated as mutations are, in particular, insertions, deletions, point mutations, inversions and polymorphisms and, particularly preferred, SNPs (single nucleotide polymorphisms).

In the context of the present invention, "epigenetic parameters" are, in particular, cytosine methylations and further chemical modifications of DNA bases of genes associated with signal transduction and sequences further required for their regulation. Further epigenetic parameters include, for example, the acetylation of histones which, however, cannot be directly analyzed using the described method but which, in turn, correlates with the DNA methylation.

In the following, the present invention will be explained in greater detail on the basis of the sequences and examples with respect to the accompanying figure without being limited thereto.

Figure 1

Figure 1 shows the hybridisation of fluorescent labelled amplicates to a surface bound oligonucleotide. Sample I being from an astrocytoma tumor sample and sample II being from an oligodendroglioma grade II tumor sample. Fluorescence at a spot shows hybridisation of the amplicate to the oligonucleotide. Hybridisation to a CG oligonucleotide denotes methylation at the cytosine position being analysed, hybridisation to a TG oligonucleotide denotes no methylation at the cytosine position being analysed. It can be seen that Sample I had a higher degree of methylation than Sample II.

Seq. ID No. 1 through Seq. ID No. 388

Sequences having odd sequence numbers (e.g., Seq. ID No. 1, 3, 5, ...) exhibit in each case sequences of the chemically pretreated genomic DNAs of different genes associated with signal transduction. Sequences having even sequence numbers (e.g., Seq. ID No. 2, 4, 6, ...) exhibit in each case the sequences of the chemically pretreated genomic DNAs of genes associated with signal transduction which are complementary to the preceding sequences (e.g., the complementary sequence to Seq. ID No.1 is Seq. ID No.2, the complementary sequence to Seq. ID No.3 is Seq. ID No.4, etc.)

Seq. ID No. 389 through Seq. ID No. 392

Seq. ID No. 389 through Seq. ID No. 392 show sequences of oligonucleotides used in Example 1.

The following example relates to a fragment of a gene associated with signal transduction, in this case, AR in which a specific CG-position is analyzed for its methylation status.

Example 1: Methylation analysis of the gene AR associated with signal transduction.

The following example relates to a fragment of the gene AR in which a specific CG-position is to be analyzed for methylation.

In the first step, a genomic sequence is treated using bisulfite (hydrogen sulfite, disulfite) in such a manner that all cytosines which are not methylated at the 5-position of the base are modified in

such a manner that a different base is substituted with regard to the base pairing behavior while the cytosines methylated at the 5-position remain unchanged.

If bisulfite solution is used for the reaction, then an addition takes place at the non-methylated cytosine bases. Moreover, a denaturing reagent or solvent as well as a radical interceptor must be present. A subsequent alkaline hydrolysis then gives rise to the conversion of non-methylated cytosine nucleobases to uracil. The chemically converted DNA is then used for the detection of methylated cytosines. In the second method step, the treated DNA sample is diluted with water or an aqueous solution. Preferably, the DNA is subsequently desulfonated at an alkaline pH value. In the third step of the method, the DNA sample is amplified in a polymerase chain reaction, preferably using a heat-resistant DNA polymerase. In the present case, cytosines of the gene AR are analyzed. To this end, a defined fragment having a length of 460 bp is amplified with the specific primer oligonucleotides GTAGTAGTAGTAGTAAGAGA (Sequence ID 389) and ACCCCCTAAA TAATTATCCT (Sequence ID No. 390). This amplificate serves as a sample which hybridizes to an oligonucleotide previously bonded to a solid phase, forming a duplex structure, for example TGTTATTTTCGAGAGAGGT (Sequence ID No. 391), the cytosine to be detected being located at position 157 of the amplificate. The detection of the hybridization product is based on Cy3 and Cy5 fluorescently labelled primer oligonucleotides which have been used for the amplification. A hybridization reaction of the amplified DNA with the oligonucleotide takes place only if a methylated cytosine was present at this location in the bisulfite-treated DNA. Thus, the methylation status of the specific cytosine to be analyzed is inferred from the hybridization product.

In order to verify the methylation status of the position, a sample of the amplificate is further hybridized to another oligonucleotide previously bonded to a solid phase. Said oligonucleotide is identical to the oligonucleotide previously used to analyze the methylation status of the sample, with the exception of the position in question. At the position to be analysed said oligonucleotide comprises a thymine base as opposed to a cytosine base i.e TGTTATTTTGAGAGAGGT (Sequence ID No. 392). Therefore, the hybridisation reaction only takes place if an unmethylated cytosine was present at the position to be analysed.

Example 2: Diagnosis of diseases associated with signal transduction

In order to relate the methylation patterns to one of the diseases associated with signal transduction, it is initially required to analyze the DNA methylation patterns of a group of diseased and of a group of healthy patients. These analyses are carried out, for example, analogously to Example 1. The results obtained in this manner are stored in a database and the CpG dinucleotides which are methylated differently between the two groups are identified. This can be carried out by determining individual CpG methylation rates as can be done, for example, in a relatively imprecise manner, by sequencing or else, in a very precise manner, by a methylation-sensitive "primer extension reaction". It is also possible for the entire methylation status to be analyzed simultaneously, and for the patterns to be compared, for example, by clustering analyses which can be carried out, for example, by a computer.

Subsequently, it is possible to allocate the examined patients to a specific therapy group and to treat these patients selectively with an individualized therapy.

Example 2 can be carried out, for example, for cancer and solid tumours.

Table 1

List of preferred genes associated with signal transduction according to the invention

Gene	Genbank Entry No. (http://www.ncbi.nlm.nih.gov)
DYRK4	Y09305
EPHA5	L36644
NEK3	Z29067
PCTK3	X66362
PRKAR1B	M65066
PRKM3	M84490 Z11696
PRKMK2	L11285
SH3D1B	U61167
ZAP70	L05148
PIK3CA	NM 006218
ADRBK1	NM 001619
AKT1	NM 005163
AKT2	NM 001626
ARHA	NM 001664
BMPR2	NM 001204
CHN1	NM 001822
CHN2	NM 004067
CLK3	NM 003992

Gene	Genbank Entry No. (http://www.ncbi.nlm.nih.gov)
CNK	NM 004073
CSK	NM 004383
CSNK1D	NM 001893
CTNNB1	NM 001904
CTNND2	NM 001332
DGKG	NM 001346
DRG2	NM 001388
DVL3	NM 004423
DYRK3	NM 003582
EFNA1	NM 004428
EPHA4	NM 004438
EPHB3	NM 004443
ERBB4	NM 005235
GRB2	NM 002086
HCK	NM 002110
AATK	NM 004920
MADH3	NM 005902
MAPKAPK2	NM 004759
MAP3K3	NM 002401
ROR1	NM 005012
ROR2	NM 004560
PDE4B	NM 002600
PDPK1	NM 002613
B56	NM 006245
PRKACG	NM 002732
PRKAG1	NM 002733
PRKAR1A	NM 002734
PRKCA	NM 002737
PRKCG	NM 002739
PRKCZ	NM 002744
PRKG1	NM 006258
MAPK1	NM 002745
MAPK10	NM 002753
MAPK13	NM 002754
MAPK6	NM 002748
MAPK9	NM 002752
PTK2	NM 005607
PTK2B	NM 004103
RGS7	NM 002924
RHOK	NM 002929
RYK	NM 002958
SFN	NM 006142
STAT1	NM 007315
STAT2	NM 003877
STK3	NM 006281
TIAM1	NM 003253

Gene	Genbank Entry No. (http://www.ncbi.nlm.nih.gov)
TTK	NM 003318
TYRO3	NM 006293
UBE1L	NM 003335
YES1	NM 005433
MAP3K12	NM 006301

Claims

1. A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated DNA of genes associated with signal transduction according to one of the sequences taken from the group of Seq. ID No.1 to Seq. ID No.388 and sequences complementary thereto.
2. A nucleic acid comprising a sequence at least 18 base pairs in length of a segment of the chemically pretreated DNA of genes associated with signal transduction according to one of the sequences according to the genes CD20 DYRK4 (Y09305),EPHA5 (L36644), NEK3 (Z29067), PCTK3 (X66362), PRKAR1B (M65066), PRKM3 (M84490) (Z11696), PRKMK2 (L11285), SH3D1B (U61167), ZAP70 (L05148), PIK3CA (NM_006218), ADRBK1 (NM_001619), AKT1 (NM_005163), AKT2 (NM_001626), ARHA (NM_001664), BMPR2 (NM_001204), CHN1 (NM_001822),CHN2 (NM_004067), CLK3 (NM_003992), CNK (NM_004073), CSK (NM_004383), CSNK1D (NM_001893), CTNNB1 (NM_001904), CTNND2 (NM_001332), DGKG (NM_001346), DRG2 (NM_001388), DVL3 (NM_004423), DYRK3 (NM_003582), EFNA1 (NM_004428), EPHA4 (NM_004438), EPHB3 (NM_004443), ERBB4 (NM_005235), GRB2 (NM_002086), HCK (NM_002110), AATK (NM_004920), MADH3 (NM_005902),MAPKAPK2 (NM_004759), MAP3K3 (NM_002401), ROR1 (NM_005012), ROR2 (NM_004560), PDE4B (NM_002600), PDPK1 (NM_002613), B56 (NM_006245), PRKACG (NM_002732), PRKAG1 (NM_002733), PRKAR1A (NM_002734), PRKCA (NM_002737), PRKCG (NM_002739), PRKCZ (NM_002744), PRKG1 (NM_006258), MAPK1 (NM_002745), MAPK10 (NM_002753), MAPK13 (NM_002754), MAPK6 (NM_002748), MAPK9 (NM_002752), PTK2 (NM_005607), PTK2B (NM_004103), RGS7 (NM_002924), RHOK (NM_002929), RYK (NM_002958), SFN (NM_006142), STAT1 (NM_007315), STAT2 (NM_003877), STK3 (NM_006281), TIAM1 (NM_003253), TTK (NM_003318), TYRO3 (NM_006293),UBE1L (NM_003335), YES1 (NM_005433), MAP3K12 (NM_006301) and sequences complementary thereto.
3. An oligomer, in particular an oligonucleotide or peptide nucleic acid (PNA)-oligomer, said oligomer comprising in each case at least one base sequence having a length of at least 9 nucleotides which hybridizes to or is identical to a chemically pretreated DNA of genes associ-

ated with signal transduction according to one of the Seq ID Nos 1 to 388 according to claim 1 or to a chemically pretreated DNA of genes according to claim 2 and sequences complementary thereto.

4. The oligomer as recited in Claim 3; wherein the base sequence includes at least one CpG dinucleotide.
5. The oligomer as recited in Claim 3; characterized in that the cytosine of the CpG dinucleotide is located approximately in the middle third of the oligomer.
6. A set of oligomers, comprising at least two oligomers according to any of claims 3 to 5.
7. A set of oligomers as recited in Claim 6, comprising oligomers for detecting the methylation state of all CpG dinucleotides within one of the sequences according to Seq. ID Nos. 1 through 388 according to claim 1 or a chemically pretreated DNA of genes according to claim 2, and sequences complementary thereto.
8. A set of at least two oligonucleotides as recited in Claim 3, which can be used as primer oligonucleotides for the amplification of DNA sequences of one of Seq. ID 1 through Seq. ID 388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to claim 2, and sequences complementary thereto and segments thereof.
9. A set of oligonucleotides as recited in Claim 8, characterized in that at least one oligonucleotide is bound to a solid phase.
10. Use of a set of oligomer probes comprising at least ten of the oligomers according to any of claims 6 through 9 for detecting the cytosine methylation state and/or single nucleotide polymorphisms (SNPs) in a chemically pretreated genomic DNA according to claim 1 or a chemically pretreated DNA of genes according to claim 2.
11. A method for manufacturing an arrangement of different oligomers (array) fixed to a carrier material for analyzing diseases associated with the methylation state of the CpG dinucleo-

tides of one of the Seq. ID 1 through Seq. ID 388 and sequences complementary thereto and/or chemically pretreated DNA of genes according to claim 2, wherein at least one oligomer according to any of the claims 3 through 5 is coupled to a solid phase.

12. An arrangement of different oligomers (array) obtainable according to claim 11.
13. An array of different oligonucleotide- and/or PNA-oligomer sequences as recited in Claim 12, characterized in that these are arranged on a plane solid phase in the form of a rectangular or hexagonal lattice.
14. The array as recited in any of the Claims 12 or 13, characterized in that the solid phase surface is composed of silicon, glass, polystyrene, aluminium, steel, iron, copper, nickel, silver, or gold.
15. A DNA- and/or PNA-array for analyzing diseases associated with the methylation state of genes, comprising at least one nucleic acid according to one of the preceding claims.
16. A method for ascertaining genetic and/or epigenetic parameters for the diagnosis and/or therapy of existing diseases or the predisposition to specific diseases by analyzing cytosine methylations, characterized in that the following steps are carried out:
 - in a genomic DNA sample, cytosine bases which are unmethylated at the 5-position are converted, by chemical treatment, to uracil or another base which is dissimilar to cytosine in terms of hybridization behavior;
 - fragments of the chemically pretreated genomic DNA are amplified using sets of primer oligonucleotides according to Claim 8 or 9 and a polymerase, the amplicates carrying a detectable label;
 - amplicates are hybridized to a set of oligonucleotides and/or PNA probes according to the Claims 6 and 7, or else to an array according to one of the Claims 12 through 15;the hybridized amplicates are subsequently detected.
17. The method as recited in Claim 16, characterized in that the chemical treatment is carried out by means of a solution of a bisulfite, hydrogen sulfite or disulfite.

18. The method as recited in one of the Claims 16 or 17, characterized in that more than ten different fragments having a length of 100 - 2000 base pairs are amplified.
19. The method as recited in one of the Claims 16 through 18, characterized in that the amplification of several DNA segments is carried out in one reaction vessel.
20. The method as recited in one of the Claims 16 through 19, characterized in that the polymerase is a heat-resistant DNA polymerase.
21. The method as recited in Claim 20, characterized in that the amplification is carried out by means of the polymerase chain reaction (PCR).
22. The method as recited in one of the Claims 16 through 21, characterized in that the labels of the amplicates are fluorescence labels.
23. The method as recited in one of the Claims 16 through 21, characterized in that the labels of the amplicates are radionuclides.
24. The method as recited in one of the Claims 16 through 21, characterized in that the labels of the amplicates are detachable molecule fragments having a typical mass which are detected in a mass spectrometer.
25. The method as recited in one of the Claims 16 through 21, characterized in that the amplicates or fragments of the amplicates are detected in the mass spectrometer.
26. The method as recited in one of the Claims 24 and/or 25, characterized in that the produced fragments have a single positive or negative net charge for better detectability in the mass spectrometer
27. The method as recited in one of the Claims 24 through 26, characterized in that detection is carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

28. The method as recited in one of the Claims 16 through 27, characterized in that the genomic DNA is obtained from cells or cellular components which contain DNA, sources of DNA comprising, for example, cell lines, biopsies, blood, sputum, stool, urine, cerebral-spinal fluid, tissue embedded in paraffin such as tissue from eyes, intestine, kidney, brain, heart, prostate, lung, breast or liver, histologic object slides, and all possible combinations thereof.
29. A kit comprising a bisulfite (= disulfite, hydrogen sulfite) reagent as well as oligonucleotides and/or PNA-oligomers according to one of the Claims 3 through 5.
30. The use of a nucleic acid according to Claims 1 or 2, of an oligonucleotide or PNA-oligomer according to one of the Claims 3 through 5, of a kit according to Claim 29, of an array according to one of the Claims 12 through 15, of a set of oligonucleotides according to one of claims 6 through 9 for the diagnosis of diseases.
31. The use of a nucleic acid according to Claims 1 or 2, of an oligonucleotide or PNA-oligomer according to one of Claims 3 through 5, of a kit according to Claim 29, of an array according to one of the Claims 12 through 15, of a set of oligonucleotides according to one of claims 6 through 9 for the therapy of diseases.

1/1

Figur 1



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(71) Applicant (*for all designated States except US*): EPIGENOMICS AG [DE/DE]; Kastanienallee 24, 10435 Berlin (DE).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): OLEK, Alexander [DE/DE]; Schröderstrasse 13/2, 10115 Berlin (DE). PIEPENBROCK, Christian [DE/DE]; Schwartzkopffstrasse 7b, 10115 Berlin (DE). BERLIN, Kurt [DE/DE]; Marienkäferweg 4, 14532 Stahnsdorf (DE).

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(74) Agents: SCHOHE, Stefan et al.; Boehmert & Boehmert, Franz-Joseph-Strasse 38, 80801 München (DE).

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(54) Title: DIAGNOSIS OF DISEASES ASSOCIATED WITH SIGNAL TRANSDUCTION

CG TG



I

CG TG



II

(57) Abstract: The present invention relates to the chemically modified genomic sequences of genes associated with signal transduction, to oligonucleotides and/or PNA-oligomers for detecting the cytosine methylation state of genes associated with signal transduction which are directed against the sequence, as well as to a method for ascertaining genetic and/or epigenetic parameters of genes associated with signal transduction.

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(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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WO 02/000926 A3

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C12Q1/68 G01N33/50 B01J19/00 C12N15/11 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EP0-Internal, WPI Data, EMBL, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL [Online] Acc.no. U07563, 23 July 1994 (1994-07-23) XP002195394	1
Y	abstract	3-31
Y	--- ASIMAKOPOULOS FOTIS A ET AL: "ABL1 methylation is a distinct molecular event associated with clonal evolution of chronic myeloid leukemia." BLOOD, vol. 94, no. 7, 1 October 1999 (1999-10-01), pages 2452-2460, XP002195393 ISSN: 0006-4971 page 2452, column 1, paragraph 1 -page 2455, column 1, paragraph 1 page 2459, column 2, paragraphs 2,3; table 1 --- -/--	3-31

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

16 April 2002

Date of mailing of the international search report

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99 28498 A (OLEK ALEXANDER ;WALTER JOERN (DE); EPIGENOMICS GMBH (DE); OLEK SVE) 10 June 1999 (1999-06-10) the whole document ---	3-31
Y	WO 95 11995 A (AFFYMAX TECH NV ;FODOR STEPHEN P A (US); GINGERAS THOMAS R (US); L) 4 May 1995 (1995-05-04) page 21, line 24 -page 33, line 31 page 92, line 22 -page 103, line 18 ---	3-31
Y	NIEMEYER C M ET AL: "DNA MICROARRAYS**" ANGEWANDTE CHEMIE, VCH VERLAGSGESELLSCHAFT, WEINHEIM, DE, vol. 38, no. 19, 1999, pages 3039-3043, XP000961724 ISSN: 0044-8249 the whole document ---	11-27
Y	REIN ET AL: "Identifying 5-methylcytosine and related modifications in DNA genomes" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 26, no. 10, 1998, pages 2255-2264, XP002143106 ISSN: 0305-1048 cited in the application page 2258, column 2, paragraph 2 -page 2261, column 2, paragraph 1 ---	16-29
Y	HERMAN J G ET AL: "METHYLATION-SPECIFIC PCR: A NOVEL PCR ASSAY FOR METHYLATION STATUS OF CPG ISLANDS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, US, vol. 93, 1 September 1996 (1996-09-01), pages 9821-9826, XP002910406 ISSN: 0027-8424 page 9821, column 1, paragraph 1 -page 9822, column 2, paragraph 1; table 1 ---	3-31
A	MELKI JOHN R ET AL: "Concurrent DNA hypermethylation of multiple genes in acute myeloid leukemia." CANCER RESEARCH, vol. 59, no. 15, 1 August 1999 (1999-08-01), pages 3730-3740, XP002196183 ISSN: 0008-5472 page 3730, column 2, paragraph 4 -page 3731, column 1, paragraph 1 abstract; table 1 -----	1,3-31

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **31(in part)**
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1,3-31 all in part

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,3-31(all in part)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claim 31 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 31(in part)

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy (claim 31)

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9928498	A	10-06-1999	DE 19754482 A1	01-07-1999
			AT 217348 T	15-05-2002
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